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(54) Title: TYPING OF MICROORGANISMS

(57) Abstract

The invention relates to a method of detecting, identifying and quantitating microorganisms, and to oligonucleotide probes for use in this method. In particular, the method relates to the typing of specific isolates of microorganisms, and discrimination between strains and allelic subtypes. The method uses amplification of the 16S-23S rRNA spacer region using a highly conserved region from the 3' end of the 16S-23S rRNA spacer region, and/or a highly conserved region from the 5' end of said region. The method enables epidemiological tracing of specific microorganism subtypes. Preferred primers are disclosed and claimed.

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TYPING OF MICROORGANISMS

     This invention relates to a method of detecting, identifying and quantitating microorganisms, and to oligonucleotide probes for use in this method. In
5   particular, the method relates to the typing of specific isolates of microorganisms, and discrimination between strains and allelic subtypes.

Background and Prior Art

     Correct identification of microorganisms is a
10   vital part of microbiological practice, and is a prerequisite for selecting the most suitable form of treatment of disease, for prevention of contamination of foods, and for prevention of cross-infection. In many cases it is important not only to identify the species of
15   organism, but also to determine the strain and serotype, or even an allelic subtype. Such identification at sub-species level is particularly important in epidemiological tracing, for example in establishing the origin of hospital-acquired (nosocomial) infection.
20   Disease caused by *Staphylococcus aureus* is most often the result of hospital-acquired infections. Strains of *S. aureus* that are resistant to the penicillinase-resistant antibiotic methicillin are now common, the first major nosocomial epidemic of a methicillin-resistant strain
25   of *S. aureus* (MRSA) having been described by Stewart and Holt (1963). The determination of whether or not isolates of *S. aureus* represent a single strain is of considerable epidemiological value in a hospital setting.

     Conventional methods of microbiological
30   identification require culturing of microorganisms in a suitable growth medium, and this entails a delay of at least 24 hours, and often much longer. The methods utilised are completely manual, and rely very heavily on the experience and skill of the microbiologist. Such
35   methods do not lend themselves to automation.

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The only alternative presently widely used is immunological identification, which usually requires the use of monoclonal antibodies. A prerequisite for such immunological identification is that the species of the
5   organism in question be known, or at least strongly suspected. Some antibodies of broad specificity are available for use in preliminary screening. While immunological methods can be automated, they are time consuming and expensive.
10 Other typing methods, which can be used for certain species only, include toxin detection, isolation of plasmids, bacteriophage, bacteriophage/bacteriocin typing systems, antibiotic susceptibility testing, protein typing by SDS-polyacrylamide gene electrophoresis, pulsed-field
15 gel electrophoresis, immunoblotting, and restriction endonuclease analysis.

The availability of molecular biological methods, including oligonucleotide probing and polymerase chain reaction (PCR), offers a means of more accurate, and more
20 rapid and precise identification. It also permits the identification of previously unknown organisms. Techniques based on analysis of DNA are more discriminating than traditional methods, and overcome the variability inherent in discriminating between strains by assays which rely upon
25 the phenotype of the target organism.

The rRNA operon, rrn, is present in varying copy number in all bacteria, with some regions highly conserved and others highly variable (Neefs et al, 1990). Consequently, when genomic DNA digested with a restriction
30 enzyme is hybridized to rRNA operons, several bands are detected (Garnier et al, 1991). The Southern hybridization of rRNA operons (ribotyping) to detect restriction fragment length polymorphisms (RFLPs) between strains has been reported in many bacterial species, including Salmonella
35 typhi strains (Altwegg et al, 1989), E. coli strains (LiPuma et al, 1989), Xanthomonas maltophilia (Bingen et al, 1991), Legionella pneumophila strains (Harrison et al,

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1992), and *Staphylococcus* species and subspecies, including
MRSA strains (Blumberg *et al*, 1992; Monzon *et al*, 1991;
Preheim *et al*, 1991; DeBuyser *et al*, 1992). However,
Southern hybridization is slow and labour intensive.

5   Although the rRNA operon has a very high genetic
stability and the length of the 16S rRNA gene is constant
in all eubacteria (Neefs *et al*, 1990), the number of rRNA
operons has been completely analyzed by Southern
hybridization or PCR of the rDNA in only a few eubacteria,
10   including *Escherichia coli*, demonstrating 7 operons (Morgan
et al, 1977), *Bacillus subtilis*, 10 operons (Loughney *et
al*, 1982), *Clostridium perfringens*, 10 operons (Garnier *et
al*, 1991), *C. difficile*, 10 operons (Gürtler, 1993) and
Mycobacterium species (Bercovier *et al*, 1986) and
15   *Mycoplasma* species (Amikam *et al*, 1984) 1 or 2 operons
respectively. The reports describing sequence data for the
16S-23S spacer region all include only a part of the total
number of rRNA operons per genome, including *E. coli*
demonstrating 5 spacers (Harvey *et al*, 1988), *B. subtilis*,
20   2 spacers (Loughney *et al*, 1982), *Pleisomonas
shigelloides*, 3 spacers (East *et al*, 1992), *Aeromonas
hydrophila*, 3 spacers (East & Collins, 1993), *Caulobacter
crescentus* (Feingold *et al*, 1985), *Acholeplasma laidlawii*
(Nakagawa *et al*, 1992) and *Enterococcus hirae*, (Sechi &
25   Daneo-Moore, 1993) 2 spacers respectively.

          International Patent Publication No. WO 91/16454
by N.B. Innogenetics S.A. describes use of hybridization
probes consisting of at least 15 nucleotides from the
spacer region between rRNA genes of non-viral organisms for
30   detection of non-viral microorganisms, particularly
bacteria. The probes are species-specific, and are
preferably 15 to 100 nucleotides of the spacer region
between the 16S and 23S rRNA genes. A separate
oligonucleotide probe is required for each microorganism
35   species.

          U.S. Patent No. 5,288,611 by Kohne describes
methods and probes for identification and quantification of

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any organism or group of organisms containing rRNA,
including previously unknown organisms. Probes specific
for individual species and for groups of related species,
and Probes hybridizing to rRNA or to tRNA are described.

5   U.S. Patent No. 5,292,874 by Milliman discloses
hybridization probes specific for *Staphylococcus aureus*
probes, which detect a unique rRNA sequence in the 23S rRNA
gene.

              Japanese Patent Publication No. 6090793 by Takara
10   Shuzo Co. Ltd. describes methods for detection of bacteria
of the genus *Lactobacillus*, by detection of a sequence in
the spacer region between the gene encoding 16S rRNA and
the gene encoding 23S rRNA.

              The disclosure of each of these patent
15   specifications is incorporated herein by reference. In
each case sequences within the spacer region were selected
on the basis of specificity for the organism from which
they were isolated, and used in hybridization assays or
polymerase chain reaction (PCR) for specific identification
20   of an organism. Each of U.S. Patent No. 5,288,611, U.S.
Patent No. 5,292,874 and Japanese Patent Publication
No. 6090793 requires a separate oligonucleotide for each
species of organism. None of these specifications mentions
the existence or number of rrn alleles, or describes a
25   method permitting differentiation of strains within a
species, or of allelic variations.

              International Patent Publication No. WO 93/11264
by E.I. Du Pont De Nemours & Company, the contents of which
are incorporated herein by reference, discloses a method
30   for identification of microorganisms by amplification of
hypervariable spacer regions between highly conserved
sequences encoding rRNA. These spacer region sequences are
amplified, using primer sequences; the same pair of primers
is used for all species of microorganisms; the sequences of
35   these primers are highly conserved among prokaryotic
organisms. The products of amplification are
characteristic of a given species. A further amplification

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step using arbitrarily primed polymerase chain reaction
(AP-PCR; also known as randomly amplified polymorphic DNA;
RAPD) is described as being able to differentiate serotype
or strains within a species. However, it is evident that
5   this method did not always enable differentiation between
strains, and even if this differentiation was achieved, the
patterns were not always clear. The conserved regions used
as primers in WO 93/11264 are designated E and A herein, as
described below. These findings have also been published
10   elsewhere (Jensen et al, 1993; Jensen & Straus, 1993). The
contents of these publications are also incorporated herein
by reference.

PCR analysis of the 16S rRNA gene has been used
to demonstrate species-specific differences (Gürtler et al,
15   1991) and strain differences (Vaneechoutte et al, 1992` in
various bacterial species. Allelic species-specific
differences within the 16S rRNA gene have been demonstrated
in clostridia (Gürtler et al, 1991). The rRNA alleles of
E. coli (Brosius et al, 1981) and B. subtilis (Loughney et
20   al, 1982) have been shown to have variable length
16S-23S rRNA spacer regions.

We have now surprisingly found that the presence
or absence of specific variable length rDNA spacer regions
varies between strains within a given microorganism
25   species. By using different specific conserved regions of
the rRNA operon, designated C and D herein (see Figure 1
below), as primers for polymerase chain reaction, and by
using modified PCR conditions, we can achieve amplification
of all alleles present in a microorganism sample, and thus
30   we can differentiate between strains in any bacterial
species, without the need for any further steps. The
patterns obtained were stable within strains on repeated
testing, using passaging either in vitro or in vivo,
permitting discrimination within and between species, and
35   designation of specific types within strains.

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Brief Description of the Drawings

Figure 1 shows the position of the oligonucleotide primers (A, B, C, D, E) used in the prior art and in the present invention. The abbreviations ile
5   and ala refer to the respective genes-encoding tRNA for isoleucine and alanine. Primers A and E are as disclosed in WO 93/11264.

Figure 2 illustrates the approaches used for the detection of rRNA alleles in *C. difficile* by Southern
10   hybridization and PCR. The hatched bars (A, B, C and D) show positions of the respective PCR products (Table 3), the shaded bar denotes the 16S rRNA gene, the solid bar denotes the 23S rRNA gene, and the line joining the 16S and 23S gene depicts the spacer regions. The *Hind*III site is
15   at position 975 of the 16S rRNA gene (Gürtler *et al*, 1991).

Figure 3 shows hybridization of PCR product B to Group II bands in genomic DNA isolated from *C. difficile* and *C. bifermentans* strains.

Lanes 1-10, *C. difficile* strains H13, H15, H16,
20   H17, H18, H19, H20, H23, 9689 and 9689, respectively;

Lanes 11-13, *C. bifermentans* strains AM312, AM360, and AM818, respectively;

Lane 14, pBR328 DNA digested with *Bg*II and *Hinf*I, labelled with photodigoxigenin;

25   Y indicates the position of an extra band visible in *C. bifermentans* products.

Figure 4 illustrates the hybridization of PCR product A to Group I and II bands in genomic DNA isolated from *C. difficile* strains.

30   Lane 1, pBR328 DNA digested with *Bg*II and *Hinf*I, labelled with photodigoxigenin;

Lanes 2-11, H24, H25, H26, H27, H28, H29, H30, H31, H32 and H33, respectively.

Figure 5 shows the detection of rRNA alleles in
35   *C. difficile* strains by Southern hybridization. The symbols, box shadings and the position of the *Hind*III site are described in the legend to Figure 2. Bands depicted as

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Group I (Figures 3 and 4) correspond to fragments 5' of the
*Hin*dIII site and Group II bands (Figures 4 and 5)
correspond to fragments 3' of the *Hin*dIII site. ★ refers
to bands which are not present in all strains.

5   Figure 6 shows the constant and variable length
regions within PCR product C amplified from *C. difficile*
strains, as demonstrated by agarose gel electrophoresis of
undigested (lanes 2-7) and *Hin*dIII-digested (lanes 10-15)
PCR product C.

10   Lane 1, pBR328 DNA digest with *Hin*fI and *Bgl*I;
          Lanes 2-7, H15, H24, H28, H30, H31 and H33,
respectively;
          Lane 8, no DNA control;
          Lane 9, pBR328 DNA digested with *Hin*fI and *Bgl*I;

15   Lanes 10-15, h13, H14, H17, H19, H23 and 630,
respectively.
          The standards are 2176, 1766, 1230, 1033, 653,
517, 453, 394, 298, 234 and 220 bp respectively.
          C_d, constant *Hin*dIII-digested;

20   V_u, variable undigested; and
          V_d, variable *Hin*dIII-digested.
          Figure 7 shows denaturing PAGE of PCR product C
amplified from strains of *C. difficile*.
          Lanes 1-8, H17, H15, H14, H13,, 6989, 630 H23 and

25   H19, respectively;
          Lane 9, no DNA PCR control
          Lanes 10-19, H33, H32, H31, H30, H29, H28, H27,
H26, H25 and H24, respectively.
          The sizes of the respective alleles are shown on

30   the left [mean ± SEM (number of determinations)]. The
molecular mass markers used (not shown) were λ DNA digested
with *Hin*dIII and *Eco*RI (947 and 831 bp bands only) and
SPPI DNA digested with *Eco*RI (1150 and 1000 bp bands only).
          Figure 8 is a dendrogram showing the

35   relationships of *C. difficile* ribotypes. Using maximum
parsimony, 50 equally parsimonious trees were found, one of
which is shown. The same ribotypes were found in each of

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the circled branches (a, b, c) for all 50 trees. The root
of the tree (C. bifermentans) had no bands in common with
any of the ribotypes.

Figure 9 shows the distribution of rRNA genes and
restriction sites in the region of interest in
Staphylococcus aureus. The solid line joining these genes
can vary in length in the same strain or in different
strains (Figure 12b). At the bottom of the diagram, the
dashed lines show positions of the PCR products C, I & J,
which were obtained using the primers R1392F and LR488,
SP1F and SP2R and SP1F and LR20F respectively (Table 8).
The dotted lines show the origins of HpaII fragments (E, F,
G and H) obtained from PCR product C. The locations of
other primer binding regions that were used to sequence
HpaII fragment E are also shown.

Figure 10 illustrates denaturing PAGE of PCR
products amplified from strains of S. aureus.

(a)   Methicillin resistant S. aureus (MRSA)
ribotype A, (lanes 1-3), and ribotype B (lanes 4-8). The
sizes of the respective alleles are shown on the right
[mean ± SEM (number of determinations)].

(b)   All MRSA ribotypes A-I (excluding C) and
penicillin-sensitive ribotype Pa (ATCC 9144).

(c)   Penicillin and methicillin sensitive
S. aureus strains.

Lanes 1-6: Ribotypes Pi, Pj, PF A(strain H11);
Lanes 9-16: B, Mi, Mh, Mi, Pi, Mh and Mj.
The sizes of the respective alleles are shown on
the right [mean ± SEM (number of determinations)]. The
molecular mass markers (lanes 7 & 8) used were λ DNA
digested with HindIII and EcoRI (1375 and 947 bp bands
only) and SPP1 DNA digested with EcoR1 (1150 and 1000 bp
respectively).

Figure 11 shows the alignments of 16S-23S spacer
sequences from S. aureus. PCR product C from S. aureus
strains (Table 9) was cloned into M13mp18RF and sequenced
with the primers listed in Table 8 and Figure 9. The

sequences were derived from the clones and isolates listed in Table 7. The sequences SA16S and SA223S were taken from Ludwig et al (1992). The alignment of rrn alleles with SA16S (a), rrnC, E, F, G, H, J, K & L (b) and rrn alleles
5   SA23S (c) is shown. The symbols refer to an identical base (.), and absent base (-), † = (rrnA, E, J, L, F), " = (clones 4, V17, V32) and S = (clones 4, V4, V8, V32, V43).

          Figure 12 is a dendrogram showing the
10   relationships between the 16S-23S alleles using the data in Figure 13b or Table 7. The same tree was obtained using either sets of data. One parsimonious tree was obtained with the program DNA PENNY from the PHYLIP package. Then, using MACCLADE, of the 16 possible rerootings, the tree
15   shown was selected because it was drawn using the longest allele (rrnA) as the root. The numerals indicate the numbers of changes/branch. The dotted lines show the clades which contain alleles without tRNA genes, and the solid lines show the clades which contain alleles with tRNA
20   genes.

Summary of the Invention

          The method of the invention avoids cumbersome steps required by previously available methods, and is suitable for testing large numbers of samples; it is also
25   amenable to automation.
          The method of the invention is particularly suitable for epidemiological studies, for example identifying the source of hospital outbreaks of antibiotic-resistant microorganisms, or tracing the source of
30   microorganisms causing contamination of foodstuffs.
          According to a first aspect, the invention provides a method of identification of microorganisms, comprising the steps of extracting and purifying DNA from a sample suspected to contain bacteria, and subjecting the
35   16S-23S rRNA spacer region of said DNA to amplification, using a first primer comprising a sequence from the 5' end

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of the 16S rRNA gene, and a second primer comprising a
sequence from the 3' end of the 23S rRNA gene, thereby
producing fragments having detectable differences in size
and number, and separating the amplified fragments.

5   Because the amplified fragments produced in the
method of the invention are of variable length, they can be
analysed directly, for example by electrophoresis; no other
experimental step, such as hybridzation, is necesary in
order to demonstrate differences between strains, although

10   in some situations a hybridization step could be
advantageous.

          The amplified products may be separated by any
method which provides sufficient resolution. We have found
that small conventional polyacrylamide gels have somewhat

15   poor resolution, and our studies have used long denaturing
polyacrylamide gels. However, the person skilled in the
art will be aware that other separation methods, such as
capillary electrophoresis or high performance liquid
chromatography, may be used.

20   Similarly, the studies described herein have
employed amplification of DNA sequences by polymerase chain
reaction. However, the skilled person will be aware that
other amplification methods are known, and may also be
used. For example, ligase chain reaction, 3SR

25   amplification, strand displacement amplification,
$Q\beta$ replicase reaction, or branched DNA signal amplification
are available (see Wolcott, 1992 for review). The skilled
person will readily be able to test these alternative
methods for suitability for use in the invention.

30   Optionally additional probes may be used, for
example comprising the sequence encoding $tRNA^{ile}$ and/or the
sequence encoding $tRNA^{ala}$.

          The sample will usually be a clinical sample such
as blood, tissue, urine, faeces, sputum etc., a food

35   sample, or an environmental sample such as a water sample
or a soil sample. Other types of samples may be used,
depending on the circumstances.

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The DNA may be extracted by any suitable method, but preferably the method is a rapid one. Extraction with guanidine hydrochloride or by boiling water followed by column purification are both suitable. In some cases,

5　particularly where clinical specimens are used, it may be advantageous to effect a preliminary purification of the sample following DNA extraction. If the nature of the bacteria sought to be tested is known, this may be carried out using monoclonal antibody methods, such as those using

10　monoclonal antibody conjugated to magnetic beads. Some broad spectrum antibodies are also available for this purpose.

While the method of the invention is specifically described with reference to *Clostridium difficile* and to

15　*Staphylococcus aureus*, it will be clearly understood that the invention is not limited to these organisms, and is applicable to any microorganism for which the sequences of the 16S rRNA gene and the 23S rRNA gene are known. These sequences enable suitable probes to be designed.

20　Preferably the primers used correspond to a highly conserved region from the 3' end of the spacer region, and to a highly conserved region from the 5' end of the spacer region respectively.

According to a second aspect, the invention

25　provides amplification primers for use in the method of the invention. As described above, these primers correspond to highly conserved regions from the 3' end of the 16S-23S rRNA sacer region and the 5' end of the 16S-23S rRNA spacer region respectively. Preferably they

30　correspond to regions from the 5' end of the 16S rRNA gene and to a region from the 3' end of the 23S rRNA gene respectively. Preferably the primers are 15 to 20 nucleotides long, since 15 nucleotides is generally considered to be a minimum length of primer for PCR, but

35　conservation is generally lost at greater than 20 nucleotides.

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In a preferred embodiment the primers are R1391F and LR488 or LR194F as herein defined. Most preferably LR488 is 15 to 19 nucleotides long, and R1391F is 15 to 18 nucleotides long. Primer C (LR488) is particularly
5 preferred, because it is more highly conserved than primer A.

We have found that in fact there are three regions in the first 520 base pairs of the 23S rRNA gene which are highly conserved through a wide variety of
10 species of bacteria and fungi, and which are useful as amplification probes in the present invention. These are summarised in Table 1.

Table 1

Conserved regions in the first 520bp of the 23S rRNA gene

Species	Position 21 to 38§ (A)	Position 188 to 208§ (B)	Position 456 to 474§ (C)
E. coli Plus strand	A-CGGTGGATGCCCTGGCA	GAACTGAAACATCTAAGTACC	CAGTACCGTGAGGGAAAGG
Complementary strand	T-GCCACCTACGGGACCGT	CTTGACTTTGTAGATTCATGG	GTCATGGCCACTCCCTTTCC
Pseudomonas aeruginosa	-...........T.......	....	....
Pseudomonas cepacia	G-T.........T....G	....	....
B.acillus subtilis	-...........T.......	..............A.	....
Bacillus stearothermophilus	-...........T.......	....	....
Staphylococcus aureus	-...........T.......	...........T.....	....
Staphylococcus carnosus	-...........T.......	...........T.....	....
Streptococcus haermophilus	-...........T......	............T...	....
Neisseria gonorrhoeae	-CA..C.......T....G	..........C....G.	....
Listeria monocytogenes	-...........T.......	....	....
Frankia sp.	-...........T.......	....	....
Mycobacterium leprae	-T..........T.......	...G.......C.T.	T...................
Rhodopseudomonas capsulata	T-T.........T.......	...G.......C..	....
Rhodobacter sphaeroides	--T.........TA.....	....	..............T.
Rum.amylophylus	-T..........TA.....	...G.....C..	A...................
Streptomyces griseus subsp.griseus	-...........T.....	...G.....C..	T..............T.

§ Numbering of nucleotides based on the *E. coli* 23S rRNA gene [Brosius, 1980 #126]
(.) refers to nucleotide identical to plus strand of *E. coli*.

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Detailed Description of the Invention

The invention will now be described by way of
reference only to the following non-limiting examples, and
to the drawings referred to above

5   **Bacterial strains and their Cultivation**

Bacterial strains used herein are listed in
Tables 2 and 3.

The identity of all strains of *Clostridium
difficile* was determined by biochemical tests (Cato et al,
10   1986) and confirmed by gas-liquid chromatography (Sutter et
al, 1985). Purified stocks were stored in cooked-meat
broth at room temperature or in glycerol broth at -20°C.
All strains were grown in brain heart infusion broth (BHI,
Gibco). The stability of ribotype patterns was tested by
15   passaging single colonies from horse blood agar plates
every 2-3 days over a 5 week period. Toxin B production by
C. difficile strains was detected by the method of
Boondeekhun *et al* (1993).

The identity of all *S. aureus* strains was
20   determined by biochemical tests (Kloos & Schleifer, 1986),
and antibiotic sensitivity tests were assessed by the agar
dilution method (break points were determined according to
NCCLS guidelines, Vol 13 No. 25, 1993). Purified stocks
were stored in glycerol broth at -70°C. All strains were
25   grown in trypticase soya broth (TSB,Oxoid). The stability
of ribotype patterns was tested by passaging single
colonies from sheep blood agar plates five times a week for
six weeks.

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Table 2

Strain	T	RT	Plasmid	Year of isolation	Source
C. difficile					
H17,H18	+	G	-	1990	
H23	+	F	-	1990	
H24	+	J	ND	1990	
H25	+	D	ND	1990	
H26	-	K	ND	1991	
H27	+	G	ND	1990	Heidelberg Repatriation Hospital, Melbourne, Australia
H28	+	L	ND	1990	
H29	+	H	ND	1991	
H30	+	M	ND	1991	
H31	+	D	ND	1991	
H32	+	N	ND	1991	
H33	+	E	ND	1991	
H14,H16,H20	+	E	+ (A)	1990	
H13	-	O	-	1990	
H14	-	P	+ (B)	1990	
H15	+	D	-	1990	
6390	+	H	ND	1993	
6048	+	G	ND	1993	
AM690	ND	Q	ND	1982	St Vincent's Hospital, Melbourne, Australia
ATCC 9689	+	R	-		American Type Culture Collection
630	+	F	+ (C)	1987	H. Hächler, Switzerland
C. bifermentans					
ATCC 638					American Type Culture Collection
AM312					Dr R. Wilkinson, University of Melbourne, Australia
AM360					

Abbreviations: T   Toxin production

                     RT   PCR-ribotype of *C. difficile* strains (Table 5)

The plasmid types are designated:

-     none detected

+   extrachromosomal bands detected (A, B and C are the different sized band paters obtained)

ND   not determined.

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Table 3

S. aureus strains

Location	Year	Number of Isolates
Methicillin-resistant		
Guildford, NCTC 10442	1960	1
New York, ATCC 33952	1981	1
Melbourne, RMH†	1982	10
UK, NCTC 11940	1982	1
UK, NCTC11939	1982	1
Melbourne, HRH	1982	6*
Ireland	1982-83	9
London, RFH	1983	8
London, NCTC 12232	1986	1
Melbourne, HRH	1988-89	7
Melbourne, HRH	1992-93	226§
Melbourne, RCH	1993	3
Penicillin-sensitive		
HRH	1992-94	14
Oxford, UK, ATCC 9144	1944	1
Bundaberg, Australia, NCTC 2669	1928	1
UK, NCTC 8532	1953	1
Methicillin-sensitive		
HRH	1992-93	31

The numbers of strains isolated from various locations at various times are shown. The strains from Ireland are listed in Townsend *et al* (1987) as WG1761-3 (plus 6 other strains) while those from RFH are listed in Townsend *et al* (1984) as WG2710, 2715, 2720 and 2724 and in Townsend *et al* (1987) as WG2716 (plus 3 other strains)

NCTC   National Collection of Type Cultures, UK
ATCC   American Type Culture Collection

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HRH	Heidelberg Repatriation Hospital
RMH	Royal Melbourne Hospital
RCH	Royal Children's Hospital, Melbourne
RFC	Royal Free Hospital, London
*	including H11, H12, H14
§	including D46
†	including H21

DNA isolation and amplification

Genomic DNA and plasmid DNA was isolated by the protocol of Gürtler *et al* (1991), except that the cell walls of *S. aureus* were disrupted by incubating the strain with 200 g lysostaphin ml^{-1} at 37°C for 5-10 min. DNA regions (Figures 1 and 9) were amplified by the protocol of Gürtler (1991), except that the reaction volume and amount of DNA were halved, and 1.25 units *Taq* polymerase (Boehringer) were used. The primers used are shown in Tables 4 and 7 below. For the amplification of M13 clone inserts in *S. aureus*, 50-100ng of single stranded M13 clone DNA was added to PCR mixtures using primers M13F and R.

Restriction enzyme analysis

For *C. difficile*, purified PCR products R907-LR507 and R1391-LR507 were digested singly or doubly with 10-15 units *Hind*III and *Cfo*I, as instructed by the manufacturer (Boehringer). Genomic DNA was digested with 30 units *Hind*III. The digested and undigested PCR products were resolved on 2% (w/v) low-gelling-temperature plus 2% (w/v) 'AR' agarose gels. The *Hind*III-digested genomic DNA was resolved on 1% (w/v) 'AR' agaorse gels.

For *S. aureus*, PCR product M13F-M13R was digested with 10-15 units *Dra*I or *Hinf*I, as instructed by the manufacturer (Boehringer). Genomic DNA was digested with 20 units *Hpa*II. The digested PCR products and genomic DNA were resolved on 2% w/v low-gelling-temperature plus 2% (w/v) 'AR' agarose gels.

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Southern hybridization

The protocol of Gürtler et al (1991) was followed. PCR products were labelled with digoxigenin.

Denaturing PAGE

5   The amplification protocol described above was followed with some modifications. The reaction volume was decreased by a factor of two and 2 µCi [α-^{32}P]dATP (DuPont or Amersham) was added. The reduction of the unlabelled dNTPs by a factor of four increased the yield of labelled

10   product. Radiolabelled DNA fragments were separated on a 0.4 mm thick, 38 cm wide and 50 cm high (Bio-Rad), 3.5% (w/v) polyacrylamide gel containing 7 M-urea (Sambrook et al, 1989). Gels were dried in a vacuum slab gel drier (Bio-Rad) for 2 h at 80°C. Autoradiographic exposure was

15   18-96 h.

DNA sequencing

Sequencing was performed by the dideoxynucleotide method of Sanger et al (1977) using the Bst DNA sequencing kit (BioRad). 7-Deaza-2'-deoxyguanosine triphosphate was

20   used to minimize band compression due to GC-rich regions. The primers used for sequencing are listed in Table 8 below and in Figure 9.

Data analysis

DNA sequences were processed and analysed by the

25   following methods. The DNASIS program (version 6; Pharmacia) was used to orient, join and edit DNA sequences. The orientation of inserts was deduced by alignment with the 16S or 23S rDNA seqences from B. subtilis (Green et al, 1985) or S. aureus (Ludwig et al, 1992). The 17 sequences

30   (fragment E, Figure 1) were aligned using CLUSTAL V (Higgins et al, 1992) and were aligned to the 16S and 23S rDNA sequences from S. aureus (Ludwig et al, 1992). Further modifications to the alignment were done using MACCLADE software (Maddison & Maddison, 1992).

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Phylogenetic analysis was done with the software package
PHYLIP using DNAPENNY (Felsenstein, 1993). The resulting
treefile was then imported into MACCLADE for further
analysis and presentation.

5   The presence or absence of PCR product C bands
was analysed as follows:
          (1)   The presence or absence of bands
(corresponding to region R1391-LR507) on autoradiograms was
analysed by using the program BioImage (Millipore). The
10   average sizes of the 16 alleles (rrnA-P) were calculated
from five separate gels ranging from 1-51 determinations
for the respective alleles. Using these sizes as internal
standards, molecular masses were assigned to respective
bands from the different strains. Twenty-four strains from
15   four gels were then compared at once. Presence or absence
of bands was scored by a 1 or 0, respectively.
          (2)   The resulting data matrix prepared from
four gels was analysed by maximum parsimony (Swofford,
1985).

20   Intense bands were reported as positive; when the
faint bands were also reported as positive the results did
not change. The resulting data matrix was analysed using
MIX and DOLLOP in the program PHYLIP.

Example 1   Ribotyping of Strains of Clostridium
25   difficile
          The DNA typing approaches used are shown in
Figure 2 and Table 4. Products A and B were hybridized to
HindIII-digested genomic DNA isolated from C. difficile and
C. bifermentans strains. Differences in HindIII sites on
30   both flanking sides of the 16S rRNA gene were sought within
and between strains. Products C and D were amplified from
C. difficile strains in an attempt to find differences in
the length of the 16S-23S spacer region within and between
strains.

Table 4

Regions of the rRNA operon amplified and their corresponding primers

PCR Product	Gene	Region amplified	16S-23S spacer	Primer code	Primer position and direction	Sequence
A	16S	15(S)-1408(S)	-	R015 R1391*	15-27(F) 1408-1391(R)	GATCCTGGCTCAG GACGGGCGGTGTGTACAA
B	16S	907(S)-1408(S)	-	R907 R1391*	907-926(F) 1408-1391(R)	AAACTCAAATGAATTGACGG GACGGGCGGTGTGTACAA
C	16S and 23S	1392(S)- 507(L)	+	R1391P*(D) LR488 (C)	1391-1408(F) 488-507(R)	TTGTACACACCGCCCGTC CCTTTCCCTCACGGTACTG
D	16S and 23S	907(S)- 507(L)	+	R907 LR488 (C)	907-926(F) 488-607(R)	AAACTCAAATGAATTGACGG CCTTTCCCTCACGGTACTG

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The products amplified cover the regions shown
where S=16S rRNA gene and L=23S rRNA gene. The sense of
the primers used is shown by R=reverse, F=forward and
*=identical region, with R1391 being the complemnt of
5   R1391F. The positions of all the primers are in regions
which are highly conserved in eubacteria (Neefs et al,
1990; Guttell & Fox, 1988). The nucleotide numbering
system is that of E. coli operon (Brosius et al, 1978).
The positions of each product are schematically represented
10   in Figure 2.

The bands detected by Southern hybridization
(ribotyping) have been divided into Groups I and II,
showing numerous Group II differences between strains and
fewer Group I differences. Ribotyping of 21 isolates of
15   C. difficile from the Heidelberg Repatriation Hospital and
one from St Vincent's Hospital, Melbourne, Australia,
produced 14 restriction fragment length polymorphism (RFLP)
types, 10 of which are shown in Figure 4. There were 10
group I bands, demonstrating 10 rRNA alleles in
20   C. difficile.

Products A and B consist only of parts of the
16S rRNA gene (Table 4). We have shown previously that the
16S rRNA gene is of constant length between alleles and
strains of C. difficile (Gürtler et al, 1991). When PCR
25   product B was hybridized to C. difficile genomic DNA, Group
II bands hybridized predominantly (Figure 3), the Group I
bands hybridized faintly, because product B included 62 bp
5' of the HindIII site (1/10 of product B). When product B
was hybridized to C. bifermentans genomic DNA digested with
30   HindIII (Figure 3), no Group I bands hybridized and an
extra band appeared (Y) due to an extra HindIII site at
position 675 of the 16S rRNA gene (Gürtler et al, 1991).
When PCR product A was used as a probe (Figure 4), the
Group I and II bands hybridized with equal intensity. The
35   orientation of the Group I and II bands is as shown in
Figure 5 because product B hybridized predominantly to
Group II bands and because the HindIII site lies 62 bp

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downstream from the 5' end of product B (Gürtler *et al*,
1991).

<u>Example 2</u>   <u>Identification of a Variable Length Region</u>
                    <u>Between the 16S and 23S rRNA Gene of</u>
5   <u>*Clostridium difficile*</u>

        From Figures 3, 4 and 5 it can be seen that the
Group II bands consist of the spacer region and part of the
23S rRNA gene. These Group II bands were of variable
length, which could be explained by the presence of either
10 a variable *Hind*III site or of an insertion within the
spacer or the beginning of the 23S rRNA gene. To determine
which possibility was correct, we amplified PCR products C
and D, both of which include the spacer regions (Table 4).
When the product C primer combinations of Table 4 were
15 used, several bands (V_u) of varying molecular masses were
obtained from each *C. difficile* strain, as shown in
Figure 6. The presence of bands varied from strain to
strain. When V_u bands were digested with *Hind*III, a band
appeared at 430 bp (C_d); this was of higher intensity than
20 the digested variable length bands, V_d. Band C_d appeared
in all the strains listed in Table 2 (results not shown).
When the product D primer combination was used, the same
*Hind*III band (C_d) was present, demonstrating that this band
contains the 23S rRNA gene from position 80-507 (Table 5).
25 The demonstration of band C_d shows that this region is of
constant length between alleles. Taken with the Southern
hybridization data of Figures 3 and 4, these results show
that the variable length regions lie between the 16S and
23S rRNA genes. The exact base pair location of the spacer
30 regions can only be determined when the separate alleles
have been sequenced.

Table 5

Variable 16S-23S rRNA spacer regions in C. difficile strains

Allele	Restriction enzyme site (position, bp)	C. difficile ribotype														Band Frequency*
		Q 1	L 1	D 3	E 4	J 1	N 1	G 4	F 2	O 1	P 1	K 1	H 2	M 1	R 1	
All	HindIII (1010)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
All	CfoI (1100†)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	

Constant region (16S rRNA)

Variable region (16S-23S spacer region)

	Q 1	L 1	D 3	E 4	J 1	N 1	G 4	F 2	O 1	P 1	K 1	H 2	M 1	R 1	Band Frequency*
rrnA	–	–	–	–	–	–	–	–	–	–	–	+	–	–	8.3
rrnB	–	–	+	+	+	+	+	+	+	–	+	+	+	+	87.5
rrnC	–	–	–	–	–	–	+	–	–	–	+	–	–	–	16.7
rrnD	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
rrnE	–	–	–	–	–	–	–	–	–	+	–	–	–	–	4.2
rrnF	+	+	–	+	+	–	–	–	–	+	–	+	+	–	33.3
rrnG	–	–	–	–	–	–	–	–	+	–	–	–	–	–	16.7
rrnH	+	–	–	–	–	–	–	–	–	–	+	–	–	–	4.2
rrnI	–	–	–	–	–	–	–	–	–	–	–	–	–	–	16.7
rrnJ	–	+	+	–	–	–	–	–	–	–	+	–	–	–	16.7
rrnK	–	–	–	–	–	–	–	+	–	–	–	–	–	–	29.2
rrnL	+	+	+	+	+	+	+	+	+	+	–	+	+	+	8.3
rrnM	+	–	–	–	–	–	+	+	+	+	+	+	–	–	100
															50

Allele	Restriction enzyme site (position, bp)	C. difficile ribotype														Band Frequency*
		Q 1	L 1	D 3	E 4	J 1	N 1	G 4	F 2	O 1	P 1	K 1	H 2	M 1	R 1	
rrnN		-	+	+	+	+	+	-	-	-	+	+	-	-	-	50
rrnO		+	+	+	-	-	-	+	+	+	+	-	+	+	+	70.8
rrnP		-	+	+	-	+	+	+	-	+	+	+	+	+	+	70.8
	Constant region (23S rRNA)															
All	HindIII (80)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	

* Calculated by dividing the number of isolates with allele rrn by the total number of isolates

† Enzyme site reported previously (Gürtler et al, 1991).

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<u>Example 3</u>   <u>Improving Resolution of Bands</u>

The resolution of the variable length bands was low, as can be seen in Figure 6, and so it was decided to increase the resolution with long denaturing polyacrylamide

5   gels. When this was done, the same amplification products, designated V_u in Figure 6 separated into between 5 and 9 bands per strain, with the presence of bands variable between strains. These results are shown in Figure 7. Each band was assigned as an allele, resulting in a total

10   of 16 alleles (A-P) of variable length. The constant length regions within the 16S and 23S genes were partially characterized, and the results are summarized in Table 5. The variability in length was due to variable length 16S-23S spacer regions between alleles.

15   PCR product C was amplified from various *C. difficile* strains and separated by denaturing PAGE (Figure 7). The presence of variable length alleles (rrnA-P) is shown. The size of each allele is shown in Figure 7. The outer limits of the constant regions are depicted by

20   restriction enzyme-cut sites (see Figures 5 and 6). The strain numbers corresponding to the ribotype are listed in Table 2. The number of isolates in each ribotype is listed below each letter. Th constant length regions were collated from results obtained in Figures 3, 4 and 6 and

25   Gürtler *et al* (1991). The variable length regions wree collated using BioImage software from Figure 7 and three other denaturing polyacrylamide gels.

<u>Example 4</u>   <u>Relationship Between Ribotypes of</u>
                  <u>Clostridium difficile</u>

30   When all of the *C. difficile* strains listed in Table 2 were analysed, 24 strains were divided into 14 ribotypes, which are also shown in Table 5. The dendrogram depicted in Figure 8 shows that 3 clusters (a, b, c) are found in all trees analysed. Within ribotype G, 2 isolates

35   were cultured from one patient at different times; within ribotype E, 3 isolates were cultured from one patient at

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different times. All other isolates which had identical patterns (ribotypes D, E, F, G and H) were from different patients.

Example 5   Stability of Band Patterns in *Clostridium*
5   *difficile*

The stability of band V_o sizes and patterns was investigated in detail by passaging five strains over a 5 week period. The alleles were scored as positive or negative by appearing visually identical and by having
10   similar calculated molecular masses. The results, illustrated in Figure 7 and Table 5, show that both the band sizes and patterns were highly reproducible in five *C. difficile* strains. The band sizes and patterns of strains H23 and 630 were reproducibly identical.
15   Product C was amplified from various *C. difficile* strains and separated by denaturing PAGE. Accumulated values (mean±SEM) taken from five separate electrophoresis runs are shown. The data include runs (PCR, DNA preparations and electrophoresis) done over a 9 month
20   period, as well as a stability testing experiment with the number of single colony passages per strain shown.

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Table 6
Stability of product C bands from *C. difficile* strains

Alleles	Strain				
	630	9689	H14 (type E)	H15 (type D)	H23 (type F)
*rrn*A	1166±3	1163±4	1164±4	1163±5	1161±4
*rrn*B	1108±2	1100±4	1106±3	1108±4	1106±3
*rrn*D					
*rrn*E			1068±2		
*rrn*F					1052±2
*rrn*G	1050±2				
*rrn*H					
*rrn*I				1008±2	
*rrn*J				992±2	
*rrn*K	978±2				975±1
*rrn*L	945±3	937±2	948±3	943±3	938±2
*rrn*M	928±3				922±2
*rrn*N			908±3	906±3	
*rrn*O	890±3	884±1		887±3	885±1
*rrn*P		851±1		853±3	
Statistics					
n	14	8	11	9	9
DNA preparations	5	5	4	4	5
Passages	14	14	10	10	14
PCR runs	10	5	4	4	6

The main finding of the present study was that the presence or absence of specific variable length rDNA spacer regions varied between *C. difficile* strains. The patterns obtained were stable within strains upon repeated testing after passaging *in vitro* and *in vivo*, allowed the designation of strains to specific types, enabled discrimination within and between species, and allowed for the easy testing of large numbers of strains. Thus the novel molecular typing method of the invention may be applied to epidemiological studies of *C. difficile*.

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Since 500 bp of the 5' end of the 23S gene was amplified, it was possible that the observed heterogeneity of PCR products was due to an insertion within the first 500 bp of the 23S gene. This possibility is supported by
5   several findings. At least one extra cleavage site has been reported in the large rRNA subunit of *Leptospira interrogans* (Hsu et al, 1990) and *Salmonella* species (Hsu et al, 1992), producing several fragments smaller than 23S and a 90 bp intervening sequence has been shown to be
10   excised during large subunit rRNA maturation (Burgin et al, 1990). The results presented in this specification show that in *C. difficile*, 430 bp 3' from position 507 of the 23S rRNA gene was of constant length and the 16S-23S spacer DNA was of variable length between alleles.

15   The 16S-23S spacer regions of *B. subtilis* (Vold, 1985) and *E. coli* (Fournier & Ozeki, 1985) contain tRNA genes which vary in length from 75-90 bp. Of the 7 *rrn* operons in *E. coli*, all contain from 1-3 tRNA genes (Brosius et al, 1981), while in *B. subtilis* operons, two
20   out of the three analysed sets of the 10 *rrn* have been shown to contain tRNA genes (Loughney et al, 1982). It is possible that the 16S-23S spacer regions in *C. difficile* characterized herein may contain tRNA genes.

Example 6   Ribotyping of Strains of *Staphylococcus*
25   *aureus*

Genomic DNA was isolated from *S. aureus* as described above, and amplified using the primers described in Table 6 and Figure 9.

Table 7

Sequencing primers

Code	Region	Position	Direction (sense)	Sequence
R1391PH‡	16S	1440-1417¶	F(+)	GGCCGGTTGTACACACCGCCCCGTC
R1391P* (D)	16S	1440-1417¶	F(+)	TTGTACACACCGCCCCGTC
SP1F	16S-23S spacer	51-70†	F(+)	ATTGTATTCAGTTTTGAATG
SP1R	16S-23S spacer	51-70†	R(-)	TTACTTACTTATCTAGTTTT
TRNAF	tRNA^{Ile}	100-120†	F(+)	ATAGCTCAGCTGGTTAGAGC
TRNAR	tRNA^{Ile}	100-120†	R(-)	GCTCTAACCAGCTGAGCTAT
SP2F	16S-23S spacer	281-300†	F(+)	AAAACGAGATAAGTAAGTAA
SP2R	16S-23S spacer	281-300†	R(-)	GTGGATGCCTTGGCACTAG
SP3F	16S-23S spacer	390-410†	F(+)	CACTCACAAGATTAATAACG
SP3R	16S-23S spacer	390-410†	R(-)	CGTTATTAATCTTGTGAGTG
LR20R	23S	24-42*	F(+)	GTGGATGCCTTGGCACTAG
LR20F	23S	24-42*	R(-)	CTAGTGCCAAGGCATCCAC
LR194F (B)	23S	194-214*	R(-)	CTTTCTTCTCCCGGGTACT
LR488 (C)	23S	502-520*	R(-)	CCTTCCCTCACGGTACTG
LR488H‡	23S	502-520*	R(-)	AACCGGCCTTCCCTCACGGTACTG
M13R	Phage M13	6205-6221§	F(+)	CAGGAAACAGCTATGAC
M13F	Phage M13	6291-6307§	R(-)	GTTTTCCCAGTCACGAC

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Numbering according to;

*, the published 23S rRNA sequence from *S. aureus* (Ludwig *et al*, 1992);

†, the aligned spacer sequence for *S. aureus* (Fig. 3);

¶, the published 16S rRNA sequence from *S. aureus* (Ludwig *et al*, 1992);

§, the published sequence for bacteriophage M13;

‖, sequence obtained by the present inventor;

‡, addition of *Hpa* II sites (underlined) at 5' end of R1391F & LR488.

Letters in brackets indicate the designations of primers as given in Figure 1. As stated above, primers A and E are as disclosed in WO 93/11264.

Using the DNA typing approach described above and the primers LR1391F, LR1392F and LR488 as shown in Figure 9, PCR product C was amplified from the *S. aureus* strains listed in Table 3. These included 281 MRSA from four geographically distinct clinical sources and 48 penicillin or methicillin sensitive strains from a single Melbourne source; several methicillin-resistant or sensitive strains from type culture collections were also used. These strains yielded various amplified products, of which only the most intense bands were considered to be alleles. In total, 15 alleles, designated rrnA to rrnO, were recognised, with 14 varying in length from 935 to 1223 bp, as shown in Figure 10. rrnO was 906 bp in length (results not shown).

From Figure 10A, it can be seen that among the strains, two ribotypes, A and B, that were highly reproducible in individual isolates were obtained, with 104 and 174 strains respectively (including 5 type strains). An additional 7 ribotypes were found among the remaining 9 MRSA strains; Figure 10b shows 8 MRSA ribotypes. Ribotype A was the major ribotype found between 1960 and 1989 in Melbourne (19/22 strains), Singapore (7/9 strains), Ireland (9/9 strains), New York (1/1 strain) and UK (12/12

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strains). After 1989, ribotype B was the major ribotype
found at the Heidelberg Repatriation Hospital; 176 were
ribotype B and 57 were ribotype A.

In contrast to the MRSA strains, the sensitive
5   strains showed considerably more variation in the presence
or absence of bands, yielding an additional 26 ribotypes
from the 48 strains studied. Figure 10c shows some of
these strains. The MRSA ribotypes A, B and I included some
of the penicillin or methicillin sensitive strains. The
10 occurrence of the alleles in the various ribotype classes
is summarized in Table 8.

PCR product C was amplified from various *S.
aureus* strains and separated by denaturing PAGE (Figure
10). The presence of variable length alleles (*rrn*A-O) is
15 shown. The size of each allele is shown in Figure 10. The
data were collated (using BioImage Software) from Figure 10
and four other denaturing polyacrylamide gels.

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Table 8

Distribution of alleles in *S. aureus* ribotypes

Ribotype	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	No. of Isolates
Methicillin sensitive																
Ma	+	·	+	·	·	·	+	·	+	·	·	·	·	·	+	2
Mb	·	·	+	·	·	·	+	+	·	+	·	+	·	·	+	1
Mc	·	·	+	·	·	+	+	+	·	+	·	+	·	·	·	1
Md	+	·	·	·	·	+	·	·	·	+	+	+	·	·	·	1
Me	·	·	+	+	+	+	·	+	·	+	+	·	·	·	·	1
Mf	·	·	+	+	+	·	+	·	·	+	+	·	·	·	·	1
Mg	·	·	+	+	+	·	·	+	·	·	·	·	·	·	·	1
Mh	·	+	+	+	+	+	·	+	·	·	·	·	·	·	·	2
Mi	+	·	+	+	·	·	+	+	·	·	·	·	·	·	·	2
Mj	·	·	+	·	·	+	+	+	+	+	+	+	·	·	·	2
Mk	·	·	+	·	·	+	·	+	·	·	·	·	·	·	·	2
Ml	+	·	·	·	·	+	·	·	·	+	·	+	·	·	·	1
Mm	·	·	+	·	+	+	·	+	·	·	·	·	·	·	·	1
Mn	+	+	·	·	·	+	·	·	+	+	·	+	·	·	·	1
Mo	+	·	+	·	·	+	·	·	+	+	·	+	·	·	·	1
Mp	+	·	+	·	·	·	·	+	·	·	·	+	·	·	·	2
Mq	+	·	+	+	·	+	+	·	+	·	·	·	·	·	·	1
Penicillin Sensitive																
Pa	·	+	+	+	·	+	·	+	·	·	·	·	·	·	·	2
Pc	+	·	+	+	+	+	·	·	·	·	·	·	·	·	·	6
Pd	+	·	+	+	+	·	+	+	·	+	·	·	·	·	·	1
Pe	·	·	+	·	·	+	·	·	·	+	·	+	·	·	·	1
Pf	·	·	+	·	·	+	·	·	·	+	·	+	·	·	·	1
Pg	·	·	+	·	·	+	+	+	·	·	·	·	·	·	·	1
Ph	·	·	+	·	·	+	+	+	·	+	·	·	·	·	·	1
P i	+	·	·	·	+	·	+	+	·	+	·	+	·	·	·	2
P j	+	·	+	·	·	·	·	+	·	·	·	·	·	·	·	1
MRSA																
A †	+	·	+	·	·	+	·	+	·	·	+	·	·	·	·	94
B §	+	·	+	·	·	+	·	+	·	+	·	·	·	·	·	178
C*	·	·	+	·	·	+	·	+	·	+	·	·	·	·	·	3
D ‡	+	·	+	·	·	+	·	+	·	·	·	+	+	·	·	3
E	+	·	+	·	·	·	+	·	·	·	·	·	·	·	·	1
F	·	·	·	+	·	·	·	·	+	·	+	·	·	·	·	1
G	+	·	+	·	·	·	·	+	·	+	·	·	·	·	·	1
H	+	·	+	·	·	+	·	+	·	·	+	·	+	·	·	1
I ¶	+	·	+	·	·	·	+	·	+	·	·	·	·	·	·	6

†   including H11; one isolate was penicillin-sensitive and another was methicillin-sensitive;

§   including D46 and H12; one isolate was methicillin-sensitive;

¶   five isolates were methicillin-sensitive;

*   including H14;

‡   including H21.

Example 7   **Stability of Ribotypes of _Staphylococcus_ _aureus_**

The stability of ribotypes A, B, C, D and Pa was investigated by 30 serial passages of strains 9144, H11, H12, H14 and H21 over a six-week period. The ribotype was assessed after every fifth passage by visual comparison with reference patterns, and was found to be stable except for strains H12 and H21. Strain H12 was identified as ribotype B at all passages except the fifth, where _rrn_L appeared, making it ribotype A. Strain H21 was originally found to be ribotype D; however during the stability experiment it was found to be ribotype A at all passages subsequently investigated (the colonies from which the DNA prepared were used completely). After plating out colonies from the original frozen stock, genomic DNA was prepared from 10 separate colonies of strain H21: in all cases the ribotype was found to be A. The instability of strains H12 and H21 could be explained by a contaminant or by the rearrangement, duplication or deletion of an _rrn_ operon to yield _rrn_L or _rrn_M respectively. This instability did not affect the typing of _S. aureus_ strains significantly, since it was an infrequent event.

The instability of _rrn_ operons has been reported in _B. subtilis_ (Widom _et al_, 1988) and _E. coli_ (Hill & Harnish, 1982). These reports show evidence for the loss of an _rrn_ operon, and there is also evidence for recombination leading to chromosomal rearrangement of _rrn_ operons (Hill & Harnish, 1982). The instability of ribotype D (strain H21) could be explained by the loss of

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rrnM giving rise to the stable ribotype A. The relative instability of ribotype B in the present study was due to the loss of *rrnL*. The frequency of such events is low [~10^{-4}; (Hill & Harnish, 1982)] and thus will have little
5   practical effect on this method.

<u>Example 8</u>   <u>Sequencing of Variable Length 16S-23S rRNA</u>
                            <u>Alleles</u>

In order to isolate and compare the variable length sequences of the 16S-23S rRNA alleles, PCR product C
10   from strains D46 (ribotype B), H11 and ATCC33952 (both ribotype A) was cleaved with *Hpa*II and the resulting fragments cloned into M13 vectors. PCR product C (Figure 1) was amplified as described above from *S. aureus* genomic DNA from strains D46, H11, and 33952 using primers
15   R1391F and LR488, R1391FH and LR488H or R1391F and LR194F (Table 7). For each strain, ten equivalent reactions were pooled, precipitated with 26% w/v polyethylene glycol in 20mM $MgCl_2$ (Paithankar & Prasad, 1991) and digested with *Hpa*II. For D46, 1 g of the *Hpa*II digested products were
20   end repaired with 4 units of T4 DNA polymerase (Boehringer), 200μM dNTPs, 33mM Tris-acetate (pH 8.0), 66mM potassium acetate, 10mM magnesium acetate, 0.5 mM dithiothreitol, 0.1mg bovine serum albumin ml^{-1} and incubated at 11°C for 30 minutes. For 33952 and H11 the
25   *Hpa*II digested products (1-25ng) were ligated directly into *Acc*I digested M13mp19RF (50ng: in a total of 10μl) and the end repaired *Hpa*II digested products (1-25ng) from strain D46 were ligated into *Sma*I digested M13mp19RF (50ng: in a total of 10 μl) with 1 unit T4 DNA ligase (Boehringer),
30   66mM Tris-HCl (pH 7.5), 5mM $MgCl_2$, 1mM dithiothreitol, 1mM ATP and incubated at room temperature overnight. The competent JM109 *E. coli* cells (50μl; Promega) were transformed with 2-3μl of the ligation mixtures according to the protocol described in Sambrook *et al* (1989). After
35   plates were incubated overnight at 37°C, bacteriophage M13 plaques were either picked off and grown in Luria Broth

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(LB) or colony hybridizations (Sambrook *et al*, 1989) to
*Hpa*II digested PCR product C labelled with digoxigenin were
performed. Positive plaques were then picked off and grown
in LB (Sambrook *et al*, 1989). Single stranded
5   bacteriophage M13 DNA was then prepared from all the
positive clones (Sambrook *et al*, 1989). To determine the
presence and size of the inserts, the single stranded DNA
from the M13 clones was used as a template in the PCR using
M13F and R primers which flank the *Sma*I and *Acc*I
10 restriction sites (Yanisch-Perron *et al*, 1985). Four
clones were isolated from D46, one containing insert E, one
containing insert F, one containing insert G and one
containing insert H. Sixteen clones, designated V2-V17
were isolated from H11; seven contained insert E, four
15 contained insert H, four contained insert G and one
contained insert F. With the sequence information of
insert (H), primer LR194F was designed so as to contain a
*Hpa*II site. For strain 33952, primers LR194F and R1391F
were used to obtain a mixture of PCR products, which were
20 digested with *Hpa*II to yield predominantly product E and
cloned into M13mp19; clones V18-V47 were isolated, and of
these 9 contained insert E. These results are summarized
in Table 9.

Table 9

The presence or absence of sequences within the nine (rrnA,C, E,F,G,H,J,K & L) 16S-23S spacer alleles sequenced

Allele	Clone	Strain isolated	GenBank no §	bp*	bp‡	bp¶	CS1 1-73	tRNA Ile 93-175	VS1 74-119	VS2 120-228	tRNA ala 176-270	VS3 232-270	CS2 248-270	VS4 271-415	VS5 416-449	VS6 450-527	VS7 530-537	CS3 538-546	VS8 547-550	Base pair changes
rrnL	V13	H11	U11775	303	603	981	+	·	·	·	·	·	·	·	·	·	·	·	·	
rrnK	V41	33952	U11785	319	616	994	+	·	+	·	·	+	+	+	·	·	·	+	·	na
4.	V4.	D46	U11773																	na
	V17.	H11	U11783																	
rrnJ	V8.	H11	U11776	335	634	1012	+	+	·	·	·	·	·	+	·	·	·	+	·	5
	V32.	33952	U11789																	
	V43	33952	U11780																	
			U11787																	
rrnH	V27	33952	U11778	362	661	1039	+	·	+	·	·	+	·	+	+	·	+	+	+	2
	V7	H11	U11788		663	1041														
rrnG	V2	H11	U11777	382	672	1050	+	+	·	·	·	·	·	+	+	·	+	+	+	na
rrnF	V30	33952	U11779	460	756	1134	+	+	·	·	·	·	·	+	+	+	+	+	+	4
	V38	33952	U11782																	
rrnE	V12	H11	U11774	469	764	1142	+	·	+	+	·	+	·	+	+	·	+	+	+	na
rrnC	V34	33952	U11781	473	774	1152	+	+	·	·	+	·	·	+	+	·	+	+	+	5
	V42	33952	U11786																	
rrnA	V40	33952	U11784	551	849	1227	+	+	·	·	+	·	·	+	+	+	+	+	+	na
Length							73	82	33	109	94	38	23	145	33	77	8	8	4	

Position

It was confirmed that the inserts were of
variable length when amplified by PCR and then digested
with *DraI* or *HinfI* (results not shown; restriction enzyme
cleavage sites are indicated in Figure 1). It was

5   determined from the first insert sequenced (clone 4) that
HinfI digested at the and of the tRNA^{ile} gene (Figures 1,
11b). All the PCR products which were digested with *HinfI*
contained a tRNA^{ile} gene, and this was subsequently
confirmed by DNA sequencing, as shown in Figure 11b.

10   The 16S-23S rDNA spacer sequences of 9 *rrn*
operons were determined from 3 methicillin resistant
S. aureus strains. The variation in 16S-23S spacer length
(303 bp to 551 bp) was accounted for by the type (tRNA^{ala}
or tRNA^{ile}) and number (one, both or none) of tRNA genes,

15   and by the presence or absence of other sequences of
unknown function.

Example 9   Designation of Alleles

          The designation of alleles set out in Table 9 was
made by direct correlation with fragment C molecular

20   weights (Figure 10A). The length of the spacer varied from
303 bp to 551 bp. The fragment E insert sequences were
aligned to the 16S, 23S and 16S-23S spacer rDNA sequences,
as shown in Figures 11a, 11c and 11b respectively. There
were only 4 base pair differences in the 16S rDNA

25   sequences, in contrast to 71 base pair differences in the
23S rDNA sequences. In the 16S-23S spacer rDNA (Figure 11b
and Table 9) there were no differences in CS1 and CS2;
however, there were striking gaps between alleles in
regions VS2, tRNA^{ala}, VS3, VS4, VS5, VS6, VS7, and VS8.

30   The tRNA^{ile} gene was present in *rrnJ,G,F,C* and A, while the
tRNA^{ala} gene was only present in *rrnA* and C. The number of
base pair differences between clones judged to be the same
allele was 5 for *rrnJ* (from strains H11, D46 and 33952,
isolated in 1982, 1992 and 1981 respectively), 2 for *rrnH*

35   (from strains 33952 and H11), 4 for *rrnF* (strain 33952) and
5 for *rrnC* (33952).

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Using phylogenetic analysis, the *rrn* alleles were divided into 3 distinct groups, which are shown in Figure 12; "a" contains tRNA[ile] and tRNA[ala], "b" contains tRNA[ile] only and "c" contains no tRNA genes.

5   To confirm the presence of variable length 16S-23S spacer regions in genomic DNA, PCR products I and J were hybridized to genomic DNA isolated from *S. aureus* isolates. Between 4 and 7 bands ranging from ~600 bp to ~850 bp were obtained for all strains, with variation

10   between strains. The results are in close agreement with the results obtained with PCR-ribotyping (Figure 10) and DNA sequencing (Figure 11 and Table 9).

The type of tRNA gene found in the 16S-23S spacer varies in number (0, 1 or 2) and combination between operon

15   and between species; *A. hydrophila* (East & Collins, 1993) and *E. coli* (Morgan *et al*, 1977) have tRNA[ala], tRNA[ile] and tRNA[glu]; *B. subtilis* (Loughney *et al*, 1982) and *C. crescentus* (Feingold *et al*, 1985) have tRNA[ile] and tRNA[ala]; *P. shigelloides* (East *et al*, 1992) has tRNA[glu];

20   *Methanococcus vanielli* (Jarsch & Böck, 1983) and *Enterococcus hirae* (Sechi & Daneo-Moore, 1993) have tRNA[ala]; and *Mycobacterium bovis* has no tRNA genes (Suzuki *et al*, 1987). The length of the spacer varies from 156 bp for *M. vanielli* (Jarsch & Böck, 1983) to 551 bp (present

25   specification). We have now found that there is intraspacer and interspacer variation of other sequences besides tRNA genes.

Our results show that variable length 16S-23S spacer regions occur in genomic DNA whose size range is

30   similar to the results obtained with PCR-ribotyping (Figure 10) and DNA sequencing (Table 9). The majority of strains presented in this study were from the Heidelberg Repatriation Hospital (274 strains of a total of 322). Among the MRSA strains, two ribotypes (A and B) that were

35   highly reproducible in individual isolates were obtained (Figure 10a), with 101 and 180 strains respectively (including 5 type strains). An additional 7 ribotypes were

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found among the remaining 9 MRSA strains (Figure 10b shows
8 MRSA ribotypes). Ribotype A was the major ribotype found
(ribotype A/total no of strains in location) between 1960
and 1989 in Melbourne (19/22), Ireland (9/9), New York
5   (1/1) and UK (12/12). After 1989, ribotype B was the major
ribotype found at the HRH (176 were ribotype B and 57 were
ribotype A). In contrast to the MRSA strains, the
sensitive strains showed considerably more variation in the
presence or absence of bands, yielding an additional 26
10   ribotypes from the 48 strains studied (Figure 10c shows
some of these strains). The MRSA ribotypes A, B and I
included some of the penicillin or methicillin sensitive
strains. The occurrence of the alleles in the various
ribotype classes is summarized in Table 7.
15   Thus we have shown that the presence or absence
of specific variable length rDNA spacer regions varies
between S. aureus strains. The patterns obtained were
mostly stable within strains upon repeated testing, allowed
the designation of strains to specific types, discriminated
20   within the species S. aureus, and allowed for the easy
testing of large numbers of strains. With these criteria
met, the molecular typing method described here is useful
for epidemiological studies of S. aureus.
             The variation in length and sequence of the
25   16S-23S spacer makes it an ideal candidate for typing of
strains and species identification which can potentially be
applied to any species of the bacterial kingdom. Our
method permits reliable, rapid identification and typing on
this basis.
30   The sequence information presented in Figure 11
is tabulated to show from which S. aureus strains the
original PCR product was isolated, which clones were
characterized, the size of the 16S-23S spacer (*), the size
of the HpaII insert (fragment E: ‡), the presence or
35   absence of sequences, with positions and lengths shown
according to numbering in Figure 13), the base pair
differences between clones for the 13 regions (CS=conserved

- 40 -

sequence, VS=variable sequence) and the GenBank accession numbers §. The size (base pairs, bp) of fragment C (¶) can be obtained by adding 331bp ($HpaII^2$ to LR520) and 47bp (R1392 to $HpaII^1$) to fragment B (‡).

5   Whereas the bacteriophage typing system was formerly the standard method for S. aureus (Williams et al, 1953), many current MRSA strains are not typable by the International Set of Phages (Richardson et al, 1988), requiring the addition of further experimental phages.

10   RFLP analysis by pulsed-field-gel electrophoresis has been shown to be more discriminating than phage typing (Schlichting et al, 1993). However, RFLP analysis relies on the stability of restriction enzyme recognition sites such that a point mutation within a site will result in a

15   different RFLP. The sequence conservation of the rrn operons (Woese, 1987) argues for the use of the 16S-23S spacer region as a more stable and direct indicator of the evolutionary divergence of S. aureus strains, and is a valuable addition to the large number of typing methods

20   available.

       It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding, various modifications and alterations to the embodiments

25   and methods described herein may be made without departing from the scope of the inventive concept disclosed in this specification.

       References cited herein are listed on the following pages, and are incorporated herein by this

30   reference.

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CLAIMS:

1.   A method of identification of microorganisms, comprising the steps of extracting and purifying DNA from a sample suspected to contain bacteria, and subjecting the
5   16S-23S rRNA spacer region of said DNA to amplification, comprising a highly conserved region from the 3' end of the 16S-23S rRNA spacer region, and/or a highly conserved region from the 5' end of said region. thereby producing fragments having detectable differences in size and number,
10   and separating the amplified fragments.

2.   A method according to Claim 1, wherein the primers used correspond to a highly conserved region from the 3' end of the spacer region, and to a highly conserved region from the 5' end of the spacer region respectively.

15   3.   A method according to Claim 1 or Claims 2, wherein the primers comprise a sequence corresponding to a region from the 5' end of the 16S rRNA gene and/or to a region from the 3' end of the 23S rRNA gene.

4.   A method according to any one of Claims 1 to 3,
20   using a first primer comprising a sequence from the 5' end of the 16S rRNA gene, and a second primer comprising a sequence from the 3' end of the 23S rRNA gene.

5.   A method according to any one of Claims 1 to 4, wherein the primers are 15 to 20 nucleotides long.

25   6.   A method according to Claim 5, wherein the primers are respectively R1391F and selected from the group consisting of LR488 and LR194F, said primers being as herein defined.

7.   A method according to Claim 6, wherein LR488 is
30   15 to 19 nucleotides long, and R1391F is 15 to 18 nucleotides long.

8.   A method according to any one of Claims 1 to 7, wherein one or more additional probes are used.

9.   A method according to Claim 8, wherein the
35   additional probe is the sequence encoding tRNA^{ile} and/or the sequence encoding tRNA^{ala}.

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10.   A method according to any one of Claims 1 to 9, wherein the amplification is performed by a method selected from the group consisting of polymerase chain reaction, ligase chain reaction, 3SR amplification, strand displacement amplification, Qβ replicase reaction, and branched DNA signal amplification.

11.   A method according to any one of Claims 1 to 9, wherein the amplified fragments are separated by a method selected from the group consisting of denaturing polyacrylamide gel electrophoresis, capillary electrophoresis, and high performance liquid chromatography.

12.   A method according to any one of Claims 1 to 11, wherein the sample is a clinical or environmental sample.

13.   A method according to any one of Claims 1 to 11, wherein the microorganism is a *Clostridium* or a *Staphylococcus*.

14.   An amplification primer reagent for use in a method according to any one of Claims 1 to 13, comprising a highly conserved region from the 3' end of the 16S-23S rRNA spacer region, and/or a highly conserved region from the 5' end of said region.

15.   An amplification primer reagent according to Claim 14, comprising a sequence corresponding to a region from the 5' end of the 16S rRNA gene and/or to a region from the 3' end of the 23S rRNA gene.

16.   An amplification primer reagent according to Claim 15, comprising a region from the 5' end of the 16S rRNA gene and a region from the 3' end of the 23S rRNA gene.

17.   An amplification primer reagent according to any one of Claims 14 to 17, wherein the primers are 15 to 20 nucleotides long.

18.   An amplification primer reagent according to Claim 16, wherein the primers are respectively R1391F and selcted from the group consisting of LR488 and LR194F, said primers being as herein defined.

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19.   An amplification primer reagent according to
Claim 18, wherein LR488 is 15 to 19 nucleotides long, and
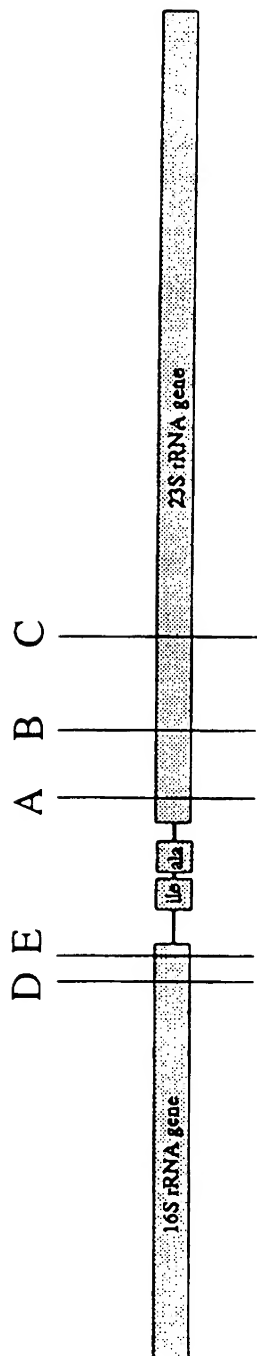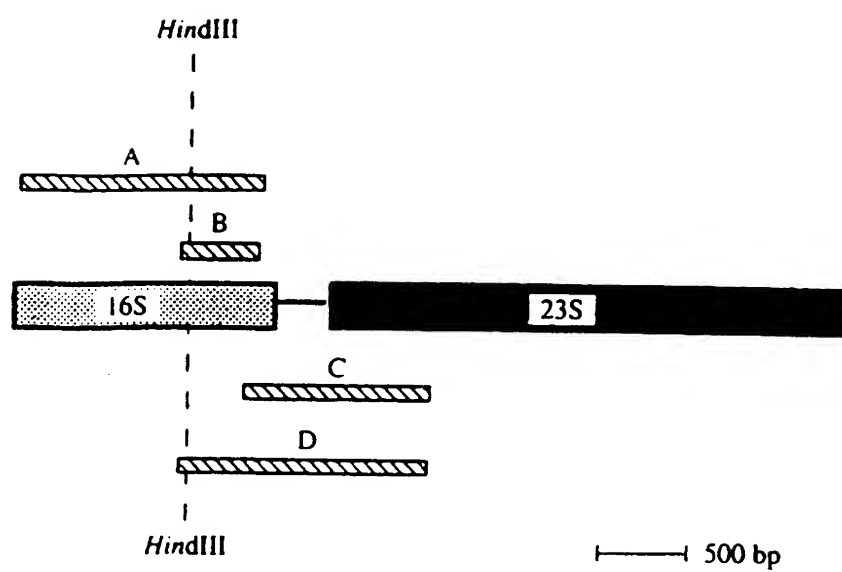R1391F is 15 to 18 nucleotides long.

FIGURE 1

FIGURE 2

FIGURE 3
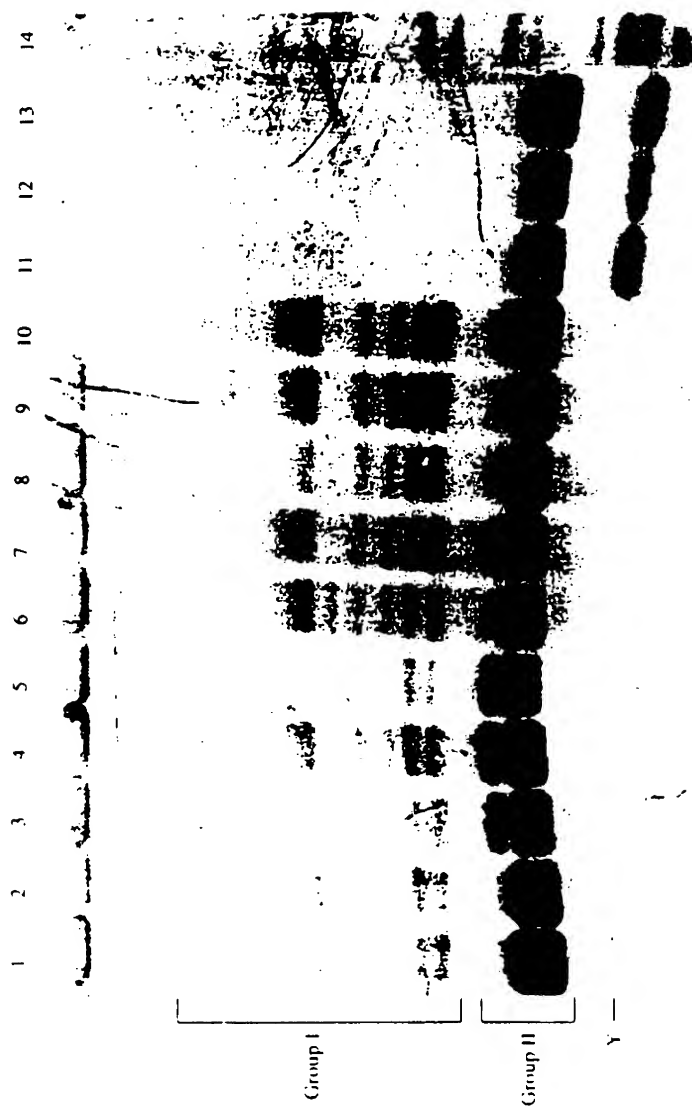SUBSTITUTE SHEET (RULE 26)

FIGURE 4

FIGURE 5
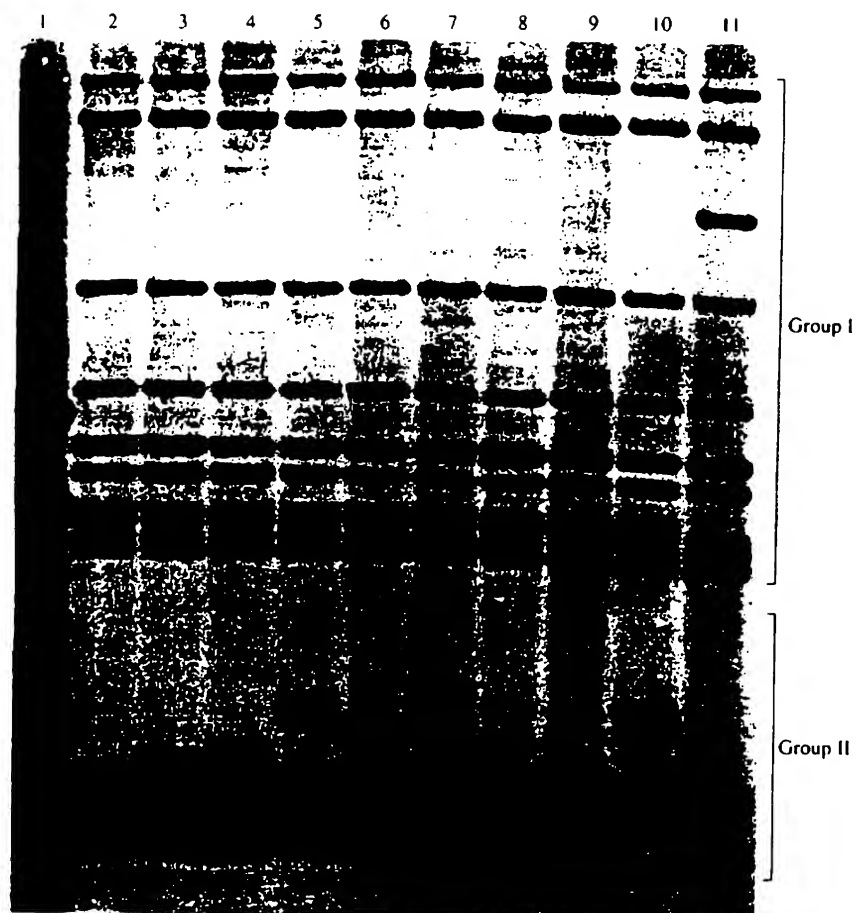
FIGURE 6

FIGURE 7

C. bifermentans

FIGURE 8

FIGURE 9

(1)

(2)

(3)

FIGURE 10

```
            1456       1466       1476       1486       1496       1506       1516       1526
SA16S  CGGTGGAGTA ACCTTTTAGG AGCTAGCCGT CGAAGGTGGG ACAAATGATT GGGGTGAA-G TCGTAACAAG GTAGCCGTAT
rrnG   .......... .......... .......... .......... .......... ..........  .......... ..........
rrnC V34 .......... .......... ..C....... .......... .......... .......-  .......... ..........
rrnC V42 .......... .......... .......... .......... .......... .......-  .......... ..........
rrnf   .......... .......... .......... .......... .......... ..........  .......... ..........
rrnH v7 .......... .......... .......... .......... .......... ......T.  .......... ..........
rrnH V27 .......... .......... .......... .......... .......... ..........  .......... ..........
rrnK   .......... .......... .......... .......-.. .......... .......-  .......... ..........


                   1536       1546       1556
SA16S       CGGAAGGTGC GGCTGGATCA CCTCCTTTCT
rrnJ,F,C,A,H,K,L .......... .......... ..........
rrnE        .......... .......-.. ..........
```

FIGURE 11a

```
              10         20         30         40         50         60         70         80
rrn J    AAGGATATAT TCGGAACATC TTCTTCAGAA GATGCGGAAT AACGTGACAT ATTGTATTCA GTTTTGAATG TTTATTTAAC
rrn G    AAGGATATAT TCGGAACATC TTCTTCAGAA GATGCGGAAT AACGTGACAT ATTGTATTCA GTTTTGAATG TTTATTTAAC
rrn C    AAGGATATAT TCGGAACATC TTCTTCAGAA GATGCGGAAT AACGTGACAT ATTGTATTCA GTTTTGAATG TTTGTT---C
rrn A    AAGGATATAT TCGGAACATC TTCTTCAGAA GATGCGGAAT AACGTGACAT ATTGTATTCA GTTTTGAATG TTTGTT---C
rrn F    AAGGATATAT TCGGAACATC TTCTTCAGAA GATGCGGAAT AACGTGACAT ATTGTATTCA GTTTTGAATG TTTATTTAAC
rrn E    AAGGATATAT TCGGAACATC TTCCTCAGAA GATGCGGAAT AACGTGACAT ATTGTATTCA GTTTTGAATG TTTATTTAAC
rrn H    AAGGATATAT TCGGAACATC TTCCTCAGAA GATGCGGAAT AACGTGACAT ATTGTATTCA GTTTTGAATG TTTATTTAAC
rrn K    AAGGATATAT TCGGAACATC TTCTTCAGAA GATGCGGAAT AATGTGACAT ATTGTATTCA GTTTTGAATG TTTATTTAAC
rrn L    AAGGATATAT TCGGAACATC TTCTTCAGAA GATGCGGAAT AATGTGACAT ATTGTATTCA GTTTTGAATG TTTATTTAAC


              90        100        110        120        130        140        150        160
rrn J    ATTCAAAAAA ATGG-GCCTA TA-GCTCAGC TGGTTAGAGC GCACGCCTGA TAAGCGTGAG GTCGGTGGTT CGAGTCCAC-
rrn G    ATTCAAA-AA ATGG-GCCTA TA-GCTCAGC TGGTTAGAGC GCACGCCTGA TAAGCGTGAG GTCGGTGGGT CGAGTCCAC-
rrn C    ATTCAAATTA ATGG-GCCTA TA-GCTCAGC TGGTTAGAGC GCACGCCTGA TAAGCGTGAG GTCGGTGGTT CGAGTCCAC-
rrn A    ATTCAAATTA ATGG-GCCTA TA-GCTCAGC TGGTTAGAGC GCACGCCTGA TAAGCGTGAG GTCGGTGGTT CGAGTCCAC-
rrn F    ATTCAAAAAA ATGG-GCCTA TA-GCTCAGC TGGTTAGAGC GCACGCCTGA TAAGCGTGAG GTCGGTGGTT CGAGTCCAC-
rrn E    ATTCAAATAT TTTTTGGTTA AA-GTGATAT TGCTTAT-GC GAGCGCTTGA CAATC-TATT CTTTTTAAAG AAAGCGGTTG
rrn H    ATTCAAATAT TTTTTGGTTA AA-GTGATAT TGCTTAT-G- ---------- ---------- ---------- ----------
rrn K    ATTCAAATAT TTTTTGGTTA AACGTGATAT TGCTTAT-G- ---------- ---------- ---------- ----------
rrn L    ATTCAAATAT TTTTTGGTTA AAG-TGATAT TGCTTAT-G- ---------- ---------- ---------- ----------


             170        180        190        200        210        220        230        240
rrn J    TTAGGCCCAC CATTA----- ---------- ---------- ---------- ---------- ---------- ----------
rrn G    TTAGGCCCAC CATTA----- ---------- ---------- ---------- ---------- ---------- ----------
rrn C    TTAGGCCCAC CATTAATTTA ATACCTATTT GGGGGCTTAG CTCAGCTGGG AGAGCGCCTG CTTTGCACGC AGGAGGTCAG
rrn A    TTAGGCCCAC CATTAATTTA ATACCTATTT GGGGGCTTAG CTCAGCTGGG AGAGCGCCTG CTTTGCACGC AGGAGGTCAG
rrn F    TTAGGCCCAC CATTA----- ---------- ---------- ---------- ---------- ---------- ----------
rrn E    TTAGACAATG CATTAA--GA AAAATTAAAG CGGAGTTTAC TTTTGTAAAT -GAGCATTTG ATTTTTTG-- -AAAATAAAG
rrn H    ---------- ---------- ---------- ---------- ---------- ---------- ---------- -AAAATAAAG
rrn K    ---------- ---------- ---------- ---------- ---------- ---------- ---------- -AAAATAAAG
rrn L    ---------- ---------- ---------- ---------- ---------- ---------- ---------- -AAAATAAAG


             250        260        270        280        290        300        310        320
rrn J    ---------- ---------- ---------T TTGTACATTG AAAACTAGAT AAGTAAGTAA AATATAGATT TTACCAAGCA
rrn G    ---------- ---------- ---------T TTGTACATTG AAAACTAGAT AAGTAAGTAA AATATAGATT TTACCAAGCA
rrn C    CGGTTCGATC CCGCTAGTCT CCACCATTAT TTGTACATTG AAAACTAGAT AAGTAAGTAA AATATAGATT TTACCAAGCA
rrn A    CGGTTCGATC CCGCTAGTCT CCACCATTAT TTGTACATTG AAAACTAGAT AAGTAAGTAA AATATAGATT TTACCAAGCA
rrn F    ---------- ---------- ---------T TTGTACATTG AAAACTAGAT AAGTAAGTAA AATATAGATT TTACCAAGCA
rrn E    CAGTATGCGA GCGCTTGACT AAAAAG-AAA TTGTACATTG AAAACTAGAT AAGTAAGTAA AATATAGATT TTACCAAGCA
rrn H    CAGTATGCGA GCGCTTGACT AAAAAG-AAA TTGTACATTG AAAACTAGAT AAGTAAGTAA AATATAGATT TTACCAAGCA
rrn K    CAGTATGCGA GCGCTTGACT AAAAAG-AAA TTGTACATTG AAAACTAGAT AAGTAAGTAA AATATAGATT TTACCAAGCA
rrn L    -------CGA GCGCTTGACT AAAAAG-AAA TTGTACATTG AAAACTAGAT AAGTAAGTAA AATATAGATT TTACCAAGCA


             330        340        350        360        370        380        390        400
rrn J    AAACCGAGTG AATAAAGAGT TTTAAATAAG CTTGAATTCA TAAGAAATAA TCGCTAGTGT TCGAAAGAAC ACTCACAAGA
rrn G    AAACCGAGTG AATAAAGAGT TTTAAATAAG CTTGAATTCA TAAGAAATAA TCGCTAGTGT TCGAAAGAAC ACTCACAAGA
rrn C    AAACCGAGTG AATAAAGAGT TTTAAATAAG CTTGAATTCA TAAGAAATAA TCGCTAGTGT TCGAAAGAAC ACTCACAAGA
rrn A    AAACCGAGTG AATAAAGAGT TTTAAATAAG CTTGAATTCA TAAGAAATAA TCGCTAGTGT TCGAAAGAAC ACTCACAAGA
rrn F    AAACCGAGTG AATAAAGAGT TTTAAATAAG CTTGAATTCA TAAGAAATAA TCGCTAGTGT TCGAAAGAAC ACTCACAAGA
rrn E    AAACCGAGTG AATAAAGAGT TTTAAATAAG CTTGAATTCA TAAGAAATAA TCGCTAGTGT TCGAAAGAAC ACTCACAAGA
rrn H    AAACCGAGTG AATAAAGAGT TTTAAATAAG CTTGAATTCA TAAGAAATAA TCGCTAGTGT TCGAAAGAAC ACTCACAAGA
rrn K    AAACCGAGTG AATAAAGAGT TTTAAATAAG CTTGAATTCA TAAGAAATAA TCGCTAGTGT TCGAAAGAAC ACTCACAAGA
rrn L    AAACCGAGTG AATAAAGAGT TTTAAATAAG CTTGAATTCA TAAGAAATAA TCGCTAGTGT TCGAAAGAAC ACTCACAAGA


             410        420        430        440        450        460        470        480
rrn J    TTAATAACGC GTTT------ ---------- ---------- ---------- ---------- ---------- ----------
rrn G    TTAATAACGC GTTTAAATCT TTTTATAAAA GAACGTAACT TCATGTTAA- ---------- ---------- ----------
rrn C    TTAATAACGC GTTTAAATCT TTTTATAAAA GAACGTAACT TCATGTTAA- ---------- ---------- ----------
rrn A    TTAATAACGC GTTTAAATCT TTTTATAAAA GAAAACGTTT AGCAGACAAT GA-GTTAAAT TATTTTAAAG CAGGAGTTTA
rrn F    TTAATAACGC GTTTAAATCT TTTTATAAAA GAAAACGTTT AGTAGACAAT GAAGTTAAAT TATTTTAAAG CAGGAGTTTA
rrn E    TTAATAACGC GTTTAAATCT TTTTATAAAA GAACGTAACT TCATGTTAA- ---------- ---------- ----------
rrn H    TTAATAACGC GTTTAAATCT TTTTA--AAA GAACGTAACT TCATGTTAA- ---------- ---------- ----------
rrn K    TTAATAACGC GTTT------ ---------- ---------- ---------- ---------- ---------- ----------
rrn L    TTAATAACGC GTTT------ ---------- ---------- ---------- ---------- ---------- ----------


             490        500        510        520        530        540        550        559
rrn J    ---------- ---------- ---------- ---------- ---------- ------CCTG TAGGA----T GGAAACATA
rrn G    ---------- ---------- ---------- ---------- ---------- CG TTTGGACTTA TAAAAATGGT GGAAACATA
rrn C    ---------- ---------- ---------- ---------- --------CG TTT-GACTTA TAAAAATGGT GGAAACATA
rrn A    CTTATGTAAA TGAGCATTTA AAATAATGAA AACGAAGCCG TATGTGAGCG TTT-GACTTA TAAAAATGGT GGAAACATA
rrn F    CTTATGTAAA TGAGCATTTA AAATAATGAA AACGAAGCCG TATGTGAGCG TTT-GACTTA TAAAAATGGT GGAAACATA
rrn E    ---------- ---------- ---------- ---------- --------CG TTT-GACTTA TAAAAATGGT GGAAACATA
rrn H    ---------- ---------- ---------- ---------- --------CG TTT-GACTTA TAAAAATGGT GGAAACATA
rrn K    ---------- ---------- ---------- ---------- ---------- ------CCTG TAGGA----T GGAAACATA
rrn L    ---------- ---------- ---------- ---------- ---------- ------CCTG TAGGA----T GGAAACATA
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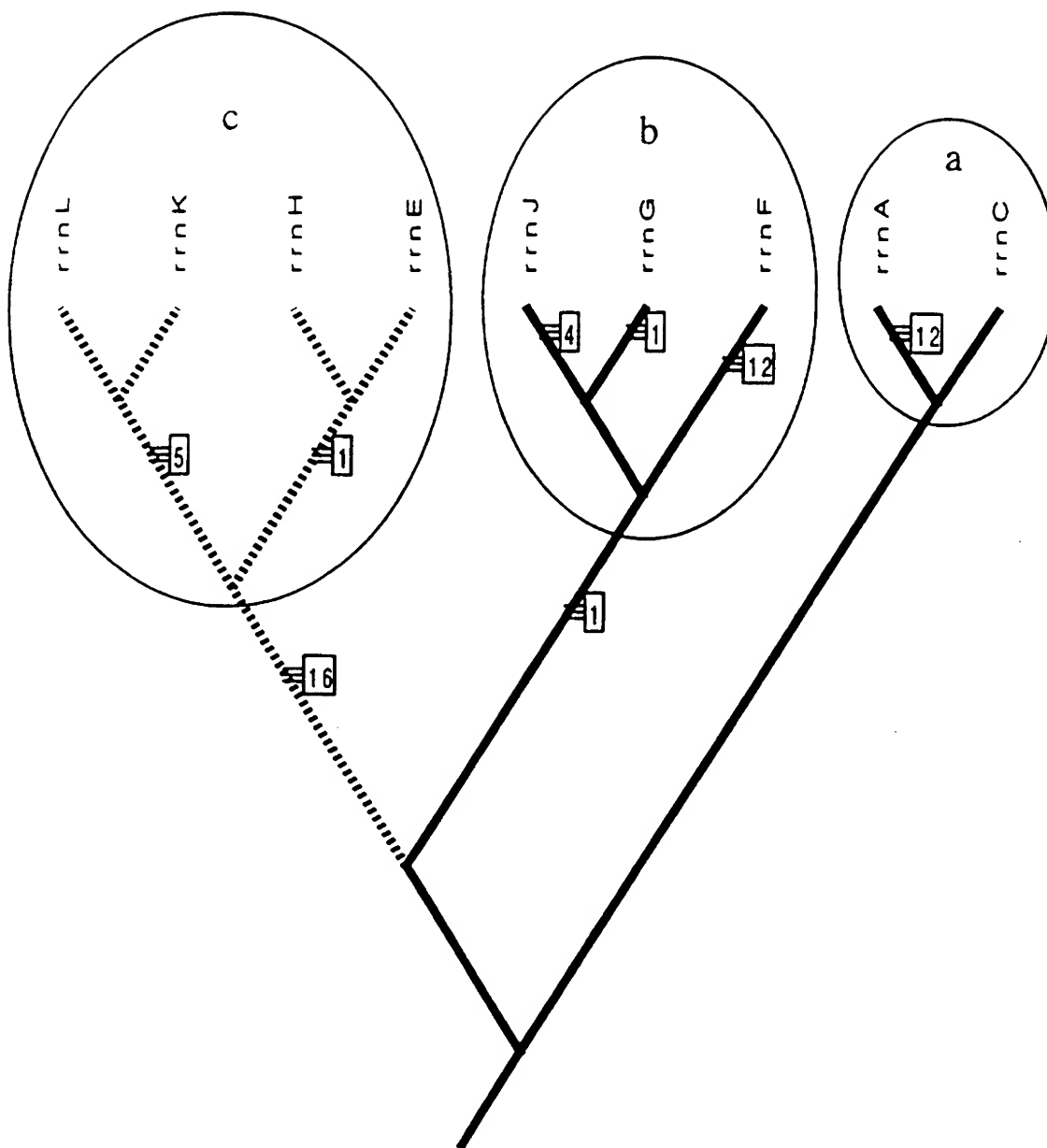
FIGURE 11b

FIGURE 11c

FIGURE 12

A.	CLASSIFICATION OF SUBJECT MATTER

Int. Cl.[6] C12Q 1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
WPAT, Chemical Abstracts. Keywords below.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Biotechnology database. Keywords below.

Electronic data base consulted during the international search (name of data base, and where practicable, search terms used)
WPAT keywords: (ribosomal(w)RNA or ribosomal(w)ribonucleic or rRNA or r(w)RNA or r(w)ribonucleic) and (C12Q or G01N or C12N)
Chem Abs & BIOT keywords: (ribosomal(w)RNA or ribosomal(w)ribonucleic or rRNA or r(w)RNA or r(w)ribonucleic) and (16s or 23s); (operon# or spacer#) and (ribonucleic or RNA); (ident: or detect: or determ: or assay#) and (pcr or polymerase(w) chain or lcr or ligase(w)chain or amplif: or replicase)

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X Y	C M Bacot & R H Reeves: "Novel tRNA Gene Organization in the 16s-23s Intergenic Spacer of the <u>Streptococcus pneumoniae</u> rRNA Gene Cluster". Journal of Bacteriology, vol 173, no. 3, pp 4234-4236, July 1991. See entire document.	1-3,14-16 4,8,9
X	T Barry et al: "The 16s/23s Ribosomal Spacer Region as a Target for DNA Probes to Identify Eubacteria". PCR Methods and Applications, vol 1, pp 51-56, (1991).	1-5,10,11, 13-17

[X] Further documents are listed in the continuation of Box C .

[X] See patent family annex.

*	Special categories of cited documents :		"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance			
"E"	earlier document but published on or after the international filing date		"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)			
"O"	document referring to an oral disclosure, use, exhibition or other means		"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"P"	document published prior to the international filing date but later than the priority date claimed			
			"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 April 1995 (07.04.95)	13 April 1995 (18 04. 95)
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No. 06 2853929	Authorized officer ROBYN PORTER Telephone No. (06) 2832318

C(Continuation).	DOCUMENTS CONSIDERED TO BE RELEVANT	
Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
	See abstract; p 51 column 3 lines 9-26; p 52 column 1 lines 2-4 and 15 - column 2 line 21, column 3 line 7; p 53 column 1 section on "PCR Amplification of the 16s/23s Spacer Sequences"; column 2 lines 8-16 and 45 - page 54 column 1 line 8; p 54 column 1 line 14 - column 3 line 8; p 55 column 1 lines 13-22; p 56 column 1 lines 4-26.	
X	EP, A1, 452 596 (N V INNOGENETICS S A) 23 October 1991 (23.10.91), priority date 18 April 1990 (18.10.90), Int Cl⁵ C12Q 1/68, C07H 21/04. See p 2 lines 1-3, p 3 lines 26-44, p 5 lines 39-45 and lines 52 to p 6 line 5, lines 51-53, p 10 lines 41-52, p 11 lines 1-48, p 14 lines 5-8, 13-17, 20-28, p 15 lines 1-13, p 16 lines 5-15, 33-36, p 18 lines 54-p 19 line 9, abstract, claims and examples.	1-5,10, 14-17
X	A K East et al: "Analysis of DNA encoding 23s rRNA and 16s-23s rRNA intergenic spacer regions from <u>Plesiomonas shigelloides</u>". FEMS Microbiology Letters, vol 95, pp 57-62 (1992). See entire document, esp. p 57 summary, p 58 section 3.2.	1,3,5, 15,17
X	T UEMORI et al: "Amplification of the 16s-23s Spacer region in rRNA Operons of Mycoplasmas by the Polymerase Chain Reaction". System. Appl. Microbiol. vol 15, pp 181-186 (1992). See Summary, p 181 column 1 last sentence, column 2 first sentence, p 182, column 1 "3. PCR", "6. Primer preparation", Results, section 1 on "Amplification of sequences", p 185 column 1 lines 6-12, column 2 lines 3-11.	1-5,10, 14-17
X	T Nakagawa et al: "<u>Acholeplasma laidlawii</u> Has tRNA Genes in the 16s-23s Spacer of the rRNA Operon". Journal of Bacteriology, vol 174, no 24, pp 8163-8165 (1992). See entire document.	1,8-10
X	B Sagredo et al: "Organization of the 16s-23s Intergenic Spacer Region of the Two rRNA Operons from <u>Thiobacillus ferrooxidans</u>". Geomicrobiology Journal, vol 10, pp 239-247 (1992). See abstract, p 239 lines 5-15, p 240 lines 1-4, 12-15, p 241 lines 8-12, 15-18, 28-29, p 244 lines 4-8.	1-5,8-11, 14-17
X	R Harasawa et al: "Detection and tentative identifaction of dominant mycoplasma species in cell cultures by restriction analysis of the 16s-23s r RNA intergenic spacer regions". Res. Microbiol. vol 144, pp 489-493 (1993). See p 490-p 491 lines 1-8.	1,2,5,8,10, 11,14,17
<u>X</u> Y	V Gurtler: "Typing of <u>Clostridium difficile</u> strains by PCR-amplification of variable length 16s-23s rDNA spacer regions". Journal of General Microbiology, vol 139, pp 3089-3097 (1993). See entire document.	<u>1-7,10-19</u> 8,9
<u>X</u> Y	A K East & M D Collins: "Molecular Characterization of DNA encoding 23s rRNA and 16s-23s rRNA intergenic spacer regions of <u>Aeromonas hydrophila</u>". FEMS Microbiology Letters, vol 106, pp 129-133 (1993). See abstract, p 129 column 2 lines 12-p 132 column 1 line 3, p 132, column 1 "PCR amplification", column 2 lines 25-28, 36-39 and Figure 2.	<u>1-3,10,14,15</u> 4,8,9,16
X	A Wada et al: "Molecular Cloning and Mapping of 16s-23s rRNA Gene Complexes of <u>Staphylococcus aureus</u>". Journal of Bacteriology, vol 175, no 22, pp 7483-7487 (1993). See p 7483, column 1 line 15-18, 30-38; p 7486, column 2 line 56-61.	1,13,14

C(Continuation).   DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
X	L M C Hall: "Are point mutations or DNA rearrangements responsible for the restriction fragment length polymorphisms that are used to type bacteria?" Microbiology, vol 140, pp 197-204 (1994). See p 198, column 1 lines 19-21 and 29-31, p 199, column 1 lines 9-12, p 201, column 2 lines 3-10, p 202, column 1 lines 1-22, column 2 last paragraph, p 203 column 1 last 2 lines - line 1 of column 2.	1-4,8-12, 14-16
X	M F Minnick et al: "Characterization of the 16s-23s rRNA intergenic spacer of Bartonella bacilliformis". Gene, vol 143, pp 149-150 (1994). See entire document especially p 150.	1,2,8-10, 14
X	S Gill et al: "Identification of variability of ribosomal DNA spacer from Pseudomonas soil isolates". Can. J. Microbiol., vol 40, pp 541-547 (1994). See p 542, column 1 last 10 lines - column 2 line 8, p 543, column 1 sections on "Oligodeoxynucleotide primers ..." and "Cloning and DNA sequence ..."; column 2 last 6 lines to p 544 column 1 line 2, column 2 lines 2-24, p 546, column 2 lines 4-19.	1-3,10-15
X	L Dolzani et al: "Typing of Staphylococcus aureus by amplification of the 16s-23s rRNA intergenic spacer sequences". FEMS Microbiology Letters vol 119, pp 167-174 (1994). See abstract, p 168, column 1 lines 28-33, column 2 last 2 lines, p 169, column 1 lines 1-3, column 2 first sentence of results, p 171, column 2 "Discussion" lines 1-9, p 172, column 1 lines 22-28.	1,3-5, 10-13,15-17
X Y	N W Kim & V L Chan: "Isolation and Characterization of the Ribosomal RNA Genes of Campylobacter jejuni". Current Microbiology, vol 19, pp 247-252 (1989). See entire document.	14 1
X Y	G de Lanversin & D T N Pillay: "Primary structure and sequence organization of the 16s-23s spacer in the ribosomal operon of soybean (Glycine max L) chloroplast DNA". Theor. Appl. Genet., vol 76, pp 443-448 (1988). See summary, p 443 column 1 last 3 lines to column 2 line 5, p 445, Table 1, column 1 line 7 - column 2 line 14, p 448, column 1 lines 6-11.	14-16 1-4,8-11
X Y	O Massenet et al: "Sequence organization of the chloroplast ribosomal of Spinacia oleracea including the 3' end of the 16s rRNA and the 5' end of the 23s rRNA". Plant Molecular Biology, vol 10, pp 53-63 (1987). See p 53, sentence spanning columns 1 and 2, p 54 column 2 lines 6-10 of "Results and discussion", p 55, column 1, lines 5-8 and 12-19, Figures 2 and 3.	14-16 1-4,8-11
X Y	M Zacharias & R Wagner: "Functional characterization of a putative inernal promoter sequence between the 16s and the 23s RNA genes within the Escherichia coli rrnB operon". Molecular Microbiology, vol 3 number 3, pp 405-410 (1989). See p 405, column 2 lines 29-32 and 35-39, p 408, column 1, "Discussion" lines 4-9 and 14-18.	14-16 1-4
Y	G de Lanversin et al: "Sequence studies on the soybean chloroplast 16s-23s rDNA spacer region". Plant Molecular Biology, vol 10, pp 65-82 (1987). See entire document.	1-4,8-11,14-16
Y	D J Lane: "16s/23s rRNA sequencing". Nucleic Acid Techniques in Bacterial Systematics, pp 115-175 (1991). See entire chapter.	3-5,15-17

C(Continuation).	DOCUMENTS CONSIDERED TO BE RELEVANT	
Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
Y	M J Wolcott: "Advances in Nucleic Acid-Based Detection Methods". Clinical Microbiology Reviews, vol 5 no 4, pp 370-386 (1992). See entire document.	10
Y	E Edmond et al: "A Ribosomal DNA Fragment of Listeria monocytogenes and Its Use as a Genus-Specific Probe in an Aqueous-Phase Hybridization Assay". Applied & Environmental Microbiology, vol 59, no 8, pp 2690-2697 (1993). See entire document.	1-4,14-16
Y	M Y L de Wit & P R Klatser: "Mycobacterium leprae isolates from different sources have identical sequences of the spacer region between the 16s and 23s ribosomal RNA genes". Microbiology, vol 140, pp 1983-1987 (1994). See p 1984, column 1 1st paragraph of "PCR", column 2, 1st paragraph of "SSCP", p 1986, column 1 lines 14-19.	1,10,11

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
EP	452596	AU	77550/91	CA	2080812	EP	525095
		HU	63463	JP	5504889	WO	9116454

END OF ANNEX